AMENDMENTS

In the Specifications

Paragraph 0002,

- Many clinicians believe that cancer is an organ-confined disease in its early stages. However, it appears that this notion is incorrect, and cancer is often a systemic disease by the time it is first detected using methods currently available. There is evidence that primary cancers begin shedding neoplastic cells into the circulation at an early disease stage prior to the appearance of clinical manifestations. Upon vascularization of a tumor, tumor cells shed into the circulation may attach and colonize at distant sites to form metastases. These circulating tumor cells (CTC) contain markers not normally found in healthy individuals' cells, thus forming the basis for diagnosis and treatment of specific carcinomas. Hence, the presence of tumor cells in the circulation can be used to screen for cancer in place of, or in conjunction with, other tests, such as mammography, or measurements of PSA. By employing appropriate ~~mononclonal~~ monoclonal antibodies directed to associated markers on or in target cells, or by using other assays for cell protein expression, or by the analysis of cellular mRNA, the organ origin of such cells may readily be determined, e.g., breast, prostate, colon, lung, ovarian or other non-hematopoietic cancers. -

Paragraph 0005

- However, whole blood is a complex body fluid containing diverse populations of cellular and soluble components capable of undergoing numerous biochemical and enzymatic reactions *in vivo* and *in vitro*, particularly on prolonged storage for more than 24hrs. Some of these reactions are related to immunoreactive destruction of circulating tumor cells that are recognized as foreign species. The patient's immune response weakens or destroys tumor cells by the normal defense mechanisms including phagocytosis and neutrophil activation. Chemotherapy similarly is intended to reduce both cell function and proliferation by inducing cell death by necrosis. Besides these external destructive factors, tumor cells damaged in a hostile environment may undergo programmed death or apoptosis. Normal and abnormal cells (including CTC) undergoing apoptosis or necrosis, have altered membrane permeabilities that allow escape of DNA,

RNA, and other intracellular components leading to formation of damaged cells, fragmented cells, cellular debris, and eventual complete disintegration. Such tumor cell debris may still bear epitopes or determinants characteristic of intact cells and can lead to spurious increases in the number of detected circulating cancer cells. Whole blood specimens from healthy individuals also have been observed to undergo destruction of labile blood cell components, herein categorized as decreased blood quality, on prolonged storage for periods of greater than 24 hours. For example, erythrocytes may rupture and release hemoglobin and produce cell ghosts. Leukocytes, particularly granulocytes, are known to be labile and diminish on storage. Such changes increase the amount of cellular debris that can interfere with the isolation and detection of rare target cells such as CTC. The combined effects of these destructive processes can substantially increase cellular debris, which is readily detectable, for instance, in flow cytometric and microscopic analyses, such as ~~CellSpotter® or CellTracks~~™ CELLSPOTTER, an image analysis device, or CELLTRACKS®, a semi-automated image analysis device, which are described in commonly-owned US Patents #5,985,153 and #6,136,182, both of which are incorporated by reference herein.-

Paragraph 0012

- In WO00/47998 ~~from Cell Works~~, two pathways are described for CTC, terminal and proliferative. Both pathways begin with an "indeterminate" cell that progresses, as determined by morphological differences, down either the terminal or proliferative pathway. A cell in the terminal pathway eventually is destroyed, and a cell in the proliferative pathway will form a new metastatic colony as a metastatic tumor. These two pathways were designed to explain morphological differences seen in patient samples.-

Paragraph 0021

- The cell may die either through apoptosis due to internal changes or messages in the cell itself. These messages may have been in the cell before intravasation or they may be received while in the blood, or it may die due to the influence of the immune system of the host, which may recognize these cells as "alien" to this environment. The results of

cellular death are identifiable in ~~CellSpotter®~~ CELLSPOTTER, an image analysis device, as enucleated cells, speckled cells or amorphous cells. These cells do not have the potential for cell division or for establishing colonies or metastases.-

Paragraph 0024

- Another dying tumor cell identifiable using ~~CellSpotter®~~ CELLSPOTTER, an image analysis device, is the amorphous cell. These cells are probably damaged during the preparation process, a sign that these may be weaker, more delicate cells but may also be the result of apoptosis or immune attack. -

Paragraph 0025

- A single epithelial malignant cell may have the potential to survive the circulation and form colonies in distant organs. These "survivor cells" appear in ~~CellSpotter®~~ CELLSPOTTER, an image analysis device, as intact cells with high nuclear material/cytoplasmic material ratio. These cells are probably undifferentiated and can potentially divide in blood and form small clumps that may extravasate in a distant capillary, where the cell may establish a new colony, or it may remain as a single cell until it extravasates, dividing once it establishes itself in the new tissue, starting this way a new colony.-

Paragraph 0033

- Decisions in follow-up on patients with known pre-malignancies: When a pap smear is diagnosed as having cells with ~~atypia~~ atypical or low-grade intraepithelial lesions, there is always the possibility that these patients have a more severe abnormality, which cells were missed as a sampling error. These patients can be colposcoped and biopsied or they may be asked to return in three months for a repeat pap smear. If the atypical cells were concurrent with a small focal area of malignant cells that did not get sampled, the patient will wait 3 months before she gets any follow-up. This may explain why some pre-malignancies seem to progress quicker than others (misdiagnoses due to sampling error, causing an artifact in statistics). These are usually explained as being a more "aggressive" pre-malignancy. ~~CellSpotter®~~ CELLSPOTTER, an image analysis device,

can be used to help in the decision tree of these patients. All patients with an abnormal pap (5-10% of the pap smears in the USA) can immediately be tested for circulating epithelial cells. Patients with positive tests should be followed-up immediately and aggressively. Patients with negative results may wait the three months for the repeat pap. This would simplify the decision making process for the physician and health professionals and help the patient trust her follow-up procedure.

Paragraph 0034

- Screening: ~~CellSpotter®~~ CELLSPOTTER, an image analysis device, image analysis may be used for screening of the general population with the condition that special, tissue specific antibodies would be used on a second test on all abnormal samples. Identification of CTC in a patient may indicate that there is a primary malignancy that has started or is starting the process of metastasis. If these cells are identified as of the tissue of origin with new markers, then organ specific tests, like CT guided fine needle aspirations (FNA) can be used to verify the presence or absence of such malignancies. Patients where a primary cannot be identified may be followed-up with repeat tests after establishing an individual base line.-

Paragraph 0036

- The methods and reagents described in this invention are used to analyze circulating tumor cells, fragments, and debris. Analysis is performed with a number of platforms, including multiparameter flow cytometry and the ~~CellSpotter®~~ CELLSPOTTER fluorescent microscopy imaging system. It is possible to mimic the damaged CTC that forms fragments and debris. Furthermore, the number of fragments and debris can be correlated back to the number of circulating tumor cells (CTC). It is also possible to inhibit further damage of CTC between the blood draw and sample processing through the use of stabilizing agents.-

Paragraph 0041

- FIG.3 – ~~CellSpotter® analysis~~ CELLSPOTTER analysis of a 7.5ml blood sample from a metastatic prostate cancer patient that was immunomagnetically enriched for tumor

cells. The lines of thumbnails correspond to the different dyes used in the staining process showing tumor candidates stained with cytokeratin PE (green) and DAPI (magenta).

Paragraph 0042

- FIG.4 – ~~CellSpotter~~®CELLSPOTTER classifications of tumor cells isolated from a single whole blood sample of a patient with metastatic prostate cancer stained with cytokeratin PE (green) and DAPI (magenta).

Paragraph 0047

- ~~CellSpotter~~® CELLSPOTTER classifications of paclitaxel treated LnCaP cells spiked into whole blood and isolated then stained with cytokeratin PE (green) and DAPI (magenta).

Paragraph 0070,

- The preferred magnetic particles for use in carrying out this invention are particles that behave as colloids. Such particles are characterized by their sub-micron particle size, which is generally less than about 200nm (0.20 microns), and their stability to gravitational separation from solution for extended periods of time. In addition to the many other advantages, this size range makes them essentially invisible to analytical techniques commonly applied to cell analysis. Particles within the range of 90-150 nm and having between 70-90% magnetic mass are contemplated for use in the present invention. Suitable magnetic particles are composed of a crystalline core of superparamagnetic material surrounded by molecules which are bonded, e.g., physically absorbed or covalently attached, to the magnetic core and which confer stabilizing colloidal properties. The coating material should preferably be applied in an amount effective to prevent non-specific interactions between biological macromolecules found in the sample and the magnetic cores. Such biological macromolecules may include carbohydrates such as sialic acid residues on the surface of non-target cells, lectins, ~~glyproteins~~glycoproteins, and other membrane components. In addition, the material should contain as much magnetic mass per nanoparticle as possible. The size of the

magnetic crystals comprising the core is sufficiently small that they do not contain a complete magnetic domain. The size of the nanoparticles is sufficiently small such that their Brownian energy exceeds their magnetic moment. As a consequence, North Pole, South Pole alignment and subsequent mutual attraction/repulsion of these colloidal magnetic particles does not appear to occur even in moderately strong magnetic fields, contributing to their solution stability. Finally, the magnetic particles should be separable in high magnetic gradient external field separators. That characteristic facilitates sample handling and provides economic advantages over the more complicated internal gradient columns loaded with ferromagnetic beads or steel wool. Magnetic particles having the above-described properties can be prepared by modification of base materials described in U.S. Patents #4,795,698, #5,597,531, and #5,698,271, each incorporated by reference herein.-

Paragraph 0079

- The term "detectably label" is used herein to refer to any substance whose detection or measurement, either directly or indirectly, by physical or chemical means, is indicative of the presence of the target bioentity in the test sample. Representative examples of useful detectable labels, include, but are not limited to the following: molecules or ions detectable based on light absorbance, fluorescence, reflectance, light scatter, phosphorescence, or luminescence properties; molecules or ions detectable by their radioactive properties; molecules or ions detectable by their nuclear magnetic resonance or paramagnetic properties. Included among the group of molecules indirectly detectable based on light absorbance or fluorescence, for example, are various enzymes which cause appropriate substrates to convert (e.g. from non-light absorbing to light absorbing molecules, or form non-fluorescent to fluorescent molecules). Analysis can be performed using any of a number of commonly used platforms, including multiparameter flow cytometry immunofluorescent microscopy, laser scanning cytometry, bright field base image analysis, capillary volumetry, spectral imaging analysis, manual cell analysis, ~~CellSpotter®~~ CELLSPOTTER analysis, ~~CellTracks~~CELLTRACKS™ analysis, and automated cell analysis.-

Paragraph 0080,

- The phrase "to the substantial exclusion of" ~~referes~~ refers to the specificity of the binding reaction between the biospecific ligand or biospecific reagent and its corresponding target determinant. Biospecific ligands and reagents have specific binding activity for their target determinant yet may also exhibit a low level of non-specific binding to other sample components.-

Paragraph 0097

-Figure 1 is a model of various CTC stages, including shedding and metastasis. Figure 1a shows these stages for cells, clusters, fragments, and debris. Figure 1b shows actual images from samples at these same stages. The images of cells clusters, fragments, and debris were taken from ~~CellSpotter®~~ CELLSPOTTER analyses of patient samples. The images of tissue samples (Origin and Metastatic sites) were taken from elsewhere (Manual of Cytology, American Society of Clinical Pathologists Press. 1983). –

Paragraph 0101

- To explore if these damaged epithelial cells and epithelial cell fragments observed in patients could be caused by apoptosis of tumor cells induced by chemotherapy, a model to mimic tumor cell death was developed. Cells of the prostate cell line LnCaP were cultured with or without paclitaxel and spiked into blood of healthy donors. The immunomagnetically selected cells of the paclitaxel treated samples analyzed by ~~CellSpotter®~~ CELLSPOTTER, an image analysis device, resembled those observed in the patient blood samples. Cells treated with paclitaxel displayed signs of apoptosis. The punctate cytokeratin staining pattern of the cells appear to correspond with a collapse of the cytoskeletal proteins (Figure 4B vs. 6B). The initiating event in the sequence resulting from the microtubule stabilizing effects of paclitaxel which in turn may activate the pro-apoptotic gene Bim that senses cytoskeletal distress. Further evidence of caspase-cleaved cytokeratin resulting from apoptosis was obtained with the M30 Cytodeath antibody (Roche Applied Science, Manheim, Germany) that recognizes an epitope of cytokeratin 18 that is only exposed following caspase cleavage in early apoptosis. Only the paclitaxel treated LnCaP cells stained with M30 and most of the

dimmer cytokeratin cells stained with M30, which would be consistent with cells undergoing apoptosis. -

Paragraph 0102

- It should be noted that a number of different cell analysis platforms can be used to identify and enumerate cells in the enriched samples. Examples of such analytical platforms are ~~Immunicon's CellSpotter® system~~ CELLSPOTTER System, a magnetic cell immobilization and analysis system, using microscopic detection for manual observation of cells described in Example 2, and the ~~CellTracks~~CELLTRACKS™ system, a more advanced automatic optical scanning system. These two analytical platforms are described in US Patents #5,876,593; #5,985,153 and #6,136,182, each of which are incorporated by reference herein as disclosing the respective apparatus and methods for manual or automated quantitative and qualitative cell analysis.-

Paragraph 0113

- For multicolor fluorescent microscopy (~~CellSpotter®~~ CELLSPOTTER, an image analysis device) analysis CD45 was conjugated to allophycocyanin (CD45-APC, Caltag, CA) whereas for flow cytometric analysis perdinin chlorophyll protein conjugated CD45 (CD45-PerCP, BDIS San Jose, CA) was used. The nucleic acid specific dye DAPI (4,6-diamidino-2-phenylindole) was used to identify and visualize the nucleus with the ~~CellSpotter®~~ CELLSPOTTER system and the nucleic acid dye used in the Procount system (BDIS, San Jose,CA) was used to identify cells by flow cytometry. After incubation, the excess staining reagents were aspirated and discarded and the captured cells were resuspended and transferred into a 12x75 mm tube for flow cytometric analysis or to a ~~CellSpotter®~~ CELLSPOTTER analysis chamber (as described in US Application 10/074,900, filed 12 February 2002, incorporated by reference herein) contained within a magnetic yoke assembly that holds the chamber between two magnets (Captivate, Molecular Probes, OR).

Paragraph 0114

- Samples were analyzed on a FACSCalibur flow cytometer equipped with a 488nm argon ion laser (BDIS, San Jose, CA). Data acquisition was performed with ~~CellQuest~~CELLQUEST analysis system (BDIS, San Jose, CA) using a threshold on the fluorescence of the nucleic acid dye. The acquisition was halted after 8000 beads or 80% of the sample was analyzed. Multiparameter data analysis was performed on the listmode data (Paint-A-Gate^{Pro}, BDIS, San Jose, CA). Analysis criteria included size defined by forward light scatter, granularity defined by orthogonal light scatter, positive staining with the PE-labeled anti-cytokeratin MAb and no staining with the PerCP-labeled anti-CD45 Mab. For each sample, the number of events present in the region typical for epithelial cells was multiplied by 1.25 to account for the sample volume not analyzed by flow cytometry.-

Paragraph 0116

- The ~~CellSpotter~~® CELLSPOTTER system consists of a microscope with a Mercury Arc Lamp mercury arc lamp, a 10X objective, a high resolution X ,Y, Z stage and a four filter cube changer. Excitation, dichroic and emission filters in each of four cubes were for DAPI 365nm/400nm/400nm, for DiOC16 480nm/495nm/510nm, for PE 546nm/560nm/580nm and for APC 620nm/660nm/700nm. Images were acquired with a digital camera connected to a digital frame grabber. The surface of the chamber is 80.2 mm^2 and 4 rows of 35 images for each of the 4 filters resulting in 560 images have to be acquired to cover the complete surface. The ~~CellSpotter~~® CELLSPOTTER acquisition program automatically determines the region over which the images are to be acquired, the number of images to acquire, the position of each image and the microscope focus to use at each position. All the images from a sample are logged into a directory that is unique to the specific sample identification. An algorithm is applied on all of the images acquired from a sample to search for locations that stain for DAPI and CK-PE. If the staining area is consistent with that of a potential tumor cell (DAPI+, CK-PE+) the software stores the location of these areas in a database. The software displays thumbnails of each of the boxes and the user can confirm that the images represented in the row are consistent with tumor cells, or stain with the leukocyte marker CD45. The

software tabulates the checked boxes for each sample and the information is stored in the database. -

Paragraph 0117

- FIG.3 shows examples of ~~CellSpotter®~~ CELLSPOTTER analysis of a blood sample from a patient with metastatic prostate cancer. Regions that potentially contain tumor cells are displayed in rows of thumbnails. The ruler in the left lower corner of the figure indicates the sizes of the thumbnails. From right to left these thumbnails represent nuclear (DAPI), cytoplasmic cytokeratin (CK-PE), control cell ($DiOC_{16}(3)$) and surface CD45 (CD45-APC) staining. The composite images shown at the left show a false color overlay of the purple nuclear (DAPI) and green cytoplasmic (CK-PE) staining. The check box beside the composite image allow the user to confirm that the images represented in the row are consistent with tumor cells and the check box beside the CD45-APC image is to confirm that a leukocyte or tumor cell stain non-specifically. In this patient sample, the software detected 2761 rows of thumbnails that demonstrated staining consistent with tumor cells. Eighteen of the 2761 rows are shown in the figure labeled 1631-1640 and 1869-1876. Rows numbered 1631, 1636, 1638, 1640, and 1873-1876 are checked off and display features of CTC defined as a size greater than 4μm, the presence of a nucleus surrounded by cytoplasmic cytokeratin staining and absence of $DiOC_{16}(3)$ and CD45 staining. Note the difference in appearance of the tumor cells: the cell in row 1638 is large and the one in row 1640 is significantly smaller. The immunophenotype of the events in rows 1634 and 1869 are consistent with tumor cells but their morphology is not consistent with intact cells. The thumbnail in row 1869 shows a large nucleus and speckled cytoplasmic due to retraction of cytoskeletal proteins consistent with apoptosis of the cell. The thumbnail in row 1634 shows a damaged cell that appears to extrude its nucleus. The thumbnail shown in row 1632 shows a cell that stains both with cytokeratin as well as CD45 and is either a tumor cell non-specifically binding to CD45 or a leukocyte non specifically staining with cytokeratin. In this instance the morphology of the cell closely resembles that of a lymphocyte. The thumbnails shown in rows 1633, 1635, 1637, 1639, 1870 and 1872 shows cytokeratin staining objects that are larger that 4μm but have no resemblance to cells. The

cytokeratin staining objects in thumbnails 1637, 1639 and 1872 are in close proximity of a leukocyte.

Paragraph 0119

- CTC were enumerated in 18 blood samples of prostate cancer patients and 27 samples from healthy individuals by both flow cytometry and ~~CellSpotter®~~ CELLSPOTTER system. The results shown in Table 1 were sorted by increasing number of intact CTC detected by ~~CellSpotter®~~the CELLSPOTTER system.

Page 27, Table 1

- **Table 1** – Enumeration of CTC by ~~CellSpotter®~~ the CELLSPOTTER system and flow cytometry in 18 blood samples of prostate cancer patients and 27 samples from healthy individuals.

Paragraph 0120

- In the ~~CellSpotter®~~ CELLSPOTTER analysis, the proportion of intact CTC clearly constituted the smallest fraction of CTC and ranged from 0% to 22% of all CTC (mean 4%). The proportion of damaged CTC ranged from 1% to 100% (mean 34%) and the CTC fragments constituted the largest portion of CTC ranging from 0% to 93% (mean 62%). The distribution of CTC over the three categories between the patients varied considerably as amplified by a lack of correlation between intact CTC and damaged CTC ($R^2 = 0.20$) and intact CTC and CTC fragments ($R^2 = 0.42$) and some correlation between damaged CTC and CTC fragments ($R^2 = 0.88$). Comparison of intact CTC by ~~CellSpotter®~~ CELLSPOTTER analysis and CTC enumerated by flow cytometry showed no significant correlation ($R^2 = 0.26$) whereas significant correlations were found between the damaged CTC and CTC by flow cytometry ($R^2 = 0.92$) and CTC fragments and CTC by flow cytometry ($R^2 = 0.93$). Comparison of the CTC detected by flow cytometry and ~~CellSpotter®~~ the CELLSPOTTER system suggests that CTC detected by flow cytometry encompass intact CTC as well as damaged CTC and to a certain extent, CTC fragments.

Paragraph 0121

-To investigate the effect of apoptosis induced by cytotoxic agents on flow cytometric and ~~CellSpotter®~~ CELLSPOTTER analysis of CTC, cells from the prostate cell line LnCaP were cultured in the presence or absence of 40nM paclitaxel for 72 hours. Following incubation, untreated LnCaP cells demonstrated a viability of >95% by trypan blue exclusion and 33% for the paclitaxel treated cells. The treated and untreated LnCaP cells were spiked into blood of healthy donors, selected by the ferrofluid methods described above, and analyzed by the ~~CellSpotter®~~ CELLSPOTTER system. In experiments in which LnCaP cells were spiked into blood that were not treated with paclitaxel greater than 95% of the LnCaP cells were classified as intact tumor cells. The morphologic appearance of the paclitaxel treated LnCaP cells showed close resemblance to that of the CTC observed in the patient samples and are shown in Figure 6. Intact LnCaP cells that survived paclitaxel treatment are shown in Figure 6A, damaged LnCaP, of which the majority show speckled cytokeratin staining, are shown in Figure 6B, and tumor fragments are shown in Figure 6C. -

Paragraph 0123

- The data shown above demonstrate that in the blood of patients with prostate cancer, CTC detected by both flow cytometry and ~~CellSpotter®~~ CELLSPOTTER system are comprised of intact cells and a variety of disintegrated cells. The apoptosis induced *in vitro* by paclitaxel suggests that the detected CTC in patient blood samples are undergoing apoptosis, necrosis, or *in vivo* damage to a varying degree caused by the treatment, mechanical damage by passage through the vascular system, or by the immune system. -